

# Determination of Point Mutational Spectra of Benzo[a]pyrene-diol Epoxide in Human Cells

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The primary goal of our research consists of developing means sufficiently sensitive to allow assessment of human exposure to environmental carcinogens. We describe here a new approach for analyzing point mutational spectra and a test for its validity and precision using cultured human cells exposed to high doses of environmental carcinogens. The approach in its present form includes *a*) treatment of independent large cultures of human cells with a carcinogen, *b*) selection of mutant cells *en masse* by 6-thioguanine resistance, *c*) amplification of sequences of interest directly from 6TG<sup>R</sup> cells using high-fidelity polymerase chain reaction, and *d*) separation of mutant sequences from nonmutant sequences using denaturing gradient gel electrophoresis. We report use of this protocol to observe induced mutational spectra in exon 3 of the *hprt* gene in cultured human cells by benzo[a]pyrene-diol epoxide (BPDE), an active form of the widely distributed environmental carcinogen benzo[a]pyrene. BPDE induced predominantly G to T transversions within this target sequence. The variation of the frequency of the mutations among independent cultures is consistent with the interpretation that each of them corresponds to a hotspot.

## Introduction

Mutagens have been shown to produce specific patterns of point mutations with regard to kinds, positions, and frequencies within a particular DNA sequence (1-3). The distribution of mutations, termed mutational spectrum, has been used as a means for studying the mechanisms of mutations (3,4) and has been proposed as a tool for detecting the causes of point mutations in humans (5). The precision and reproducibility of mutational spectra are, however, a function of the number of mutants per base analyzed within the target sequences investigated. For instance, an estimated 10 mutants analyzed per base within a target sequence would be expected to create precise and reproducible mutational spectra (6). Previously, mutational spectra have been investigated by cloning and sequencing individual mutants, and most of these studies have involved analysis of only a few mutants. In order to observe mutational spectra with a high degree of precision and reproducibility, we propose an alternative method, which includes selection of mutants *en masse*, amplifying desired DNA sequences directly from mixed mutant cells using the polymerase chain reaction (PCR) (7) with sufficiently high fidelity (8), and separation of

mutant sequences from nonmutant sequences or wild-type by denaturing gradient gel electrophoresis (DGGE) (9). The frequency of each mutant in the gel can then be estimated and the kinds and positions of mutations determined by sequencing DNA isolated from individual mutant bands appearing on the gel. This protocol has already allowed us to analyze point mutational spectra for several mutagens in the cultured human cell line TK6, including the carcinogen benzo[a]pyrene-diol epoxide (BPDE). The validity and precision of this new approach for observing mutational spectra are evaluated by comparing our data with those previously reported on other systems using conventional clone-by-clone analysis.

## Experimental Design and Results

### Characteristics of Cell Samples

For our approach to be useful in obtaining mutational spectra in human cells, a number of criteria must be met. First, the original cell sample must contain a sufficiently large number of mutant target sequences to constitute a spectrum. Second, mutant sequences must be recoverable for analysis at the DNA sequence level, in order to test the quality and accuracy of the information provided.

In order to obtain mutational spectra with a high degree of precision, a sufficiently large number of original mutants is needed. For this reason, depending on the type of chemical studied, the number of cells treated with a chemical should be large enough to provide at least 10 mutants for each base-pair of the target DNA sequence. For instance, to observe mutational spectra at the level of the *hprt* gene, which consists of about 1000 bp of coding

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exons and flanking intron sequences on which mutation occurrence may result in 6-thioguanine-resistant (6TG<sup>R</sup>) mutants, at least  $10^4$  6TG<sup>R</sup> mutants are needed. With BPDE, we observed that the 6TG<sup>R</sup> mutant fraction was about  $10^{-4}$  under optimal treatment conditions, producing a surviving cell fraction of about 60%. We therefore needed at least  $2 \times 10^8$  cells for treatment to give rise to an original 12,000 6TG<sup>R</sup> mutants, which would be further grown and harvested for DNA analysis.

## Characteristics of Target DNA Sequences

In this study, the kinds, positions, and frequencies of BPDE-induced mutations were examined within the 184-bp exon 3 sequence which represents about 28% of the total coding region of the *hprt* gene. To be suitable for analysis by DGGE, a DNA sequence must contain two contiguous domains: a high-temperature and a low-temperature melting domain (9). Figure 1 shows the melting profile of the 184-bp exon 3 sequence and its flanking

intron sequences and the positions of the primers used to amplify desired segments of this sequence for analysis by DGGE. Exon 3 already contains a natural 80-bp high-temperature domain contiguous to a 104-bp low-temperature melting domain. Therefore, this sequence can be used directly as a target for analysis of mutations occurring within the low-temperature melting domain, while the high-melting domain is used as a clamp (9). To detect mutations within the remaining 80-bp high-temperature melting domain, an artificial high-temperature melting domain was added by PCR using a primer carrying a short G/C-rich sequence or clamp (10,11). The resultant fragment (180-bp, Fig. 1) can now be used as a target to analyze mutations within the natural 80-bp high-temperature melting domain of exon 3.

## Sensitivity of Protocol for Detecting Mutant Sequences in a Wild-Type Background

The DNA polymerase used to catalyze PCR makes errors at a rate that varies with the type of DNA polymerase used (8,12). In our protocol for observing point mutational spectra, the background of polymerase-induced mutant sequences is a limiting factor for detecting pre-existing mutant sequences present as only a small fraction in the 6TG<sup>R</sup> mixed mutant cells. In order to probe this limit we carried out reconstruction experiments (Fig. 2). *hprt* Exon 3 was amplified using modified T7 DNA polymerase from samples of *hprt* wild-type TK6 cells containing known fractions of an exon 3 mutant cell (*hprt*-Munich). Modified T7 DNA polymerase was used because it is very efficient and is of sufficiently high fidelity for our protocol (8). In the DGGE analysis shown in Figure 2, a mutant fraction of  $10^{-2}$  is clearly detected, a mutant fraction of  $10^{-3}$  appears as faint band, and a mutant fraction of  $10^{-4}$  is completely obscured by the DNA polymerase noise.

## DGGE Analysis of BPDE-induced Mutant Sequences in 6TG<sup>R</sup> Cells

After amplification, the PCR products were boiled and reannealed. In this way, each mutant sequence was expected to hybridize with the wild-type sequence present in excess in the amplified DNA and to form two mutant/wild-type heteroduplexes. The latter each contain a mismatch and are thus less stable than the original mutant homoduplex; therefore, when migrated through a DGG, they would separate from the wild-type homoduplex as two bands in lower denaturing concentrations of the gel.

As an example, Figure 3 shows DGGE analysis of the 180-bp fragment containing the natural 80-bp high-melting domain of exon 3 (Fig. 1) amplified from independent cultures of 6TG<sup>R</sup> cells with or without BPDE treatment. The two bands that appear for each of the 6TG<sup>R</sup> untreated cells (indicated by arrows in lanes 2 and 5) represent background mutants in the stock cultures. After BPDE treatment, a series of additional bands appeared in all four independent experiments (indicated by arrows in

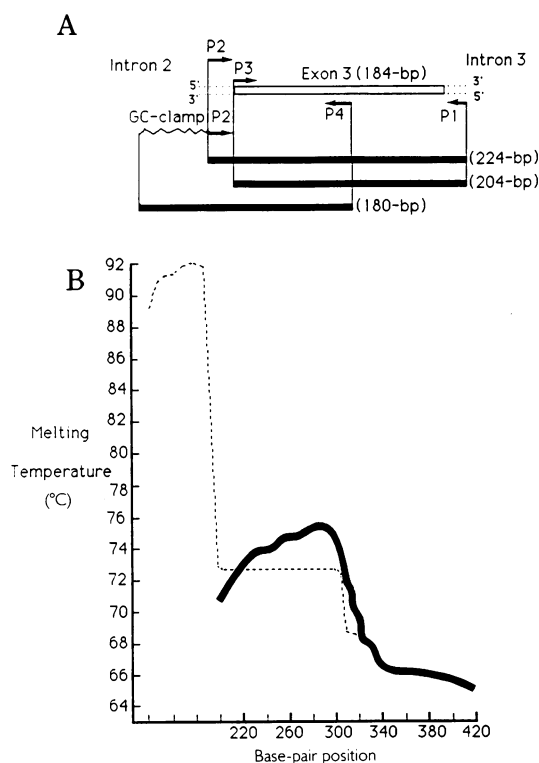


FIGURE 1. Positions of primers and melting map of exon 3 of the human *hprt* gene. (A) Positions of primers (P) used to amplify exon 3 from genomic DNA to detect mutations within the entire exon 3 sequence. Five primers were used: P1 and P2 are complementary to the introns immediately flanking the 3' and 5' ends of exon 3, respectively. Internal primer P3, adjacent to primer P2, is complementary to the 5' end of exon 3. Internal primer P4 is complementary to the sequence extending from bp 308 to bp 324. The P2-GC-clamp represents P2 extended by a 54-bp high-melting sequence or clamp (11). The expected size of polymerase chain reaction products using these primers are indicated as thick lines. (B) Melting map of the entire exon 3 sequence extending from primers P1 to P2 (for the 224-bp fragment) and of the natural 80-bp high-temperature melting domain after extension with an artificial high-temperature melting domain (for the 180-bp fragment).

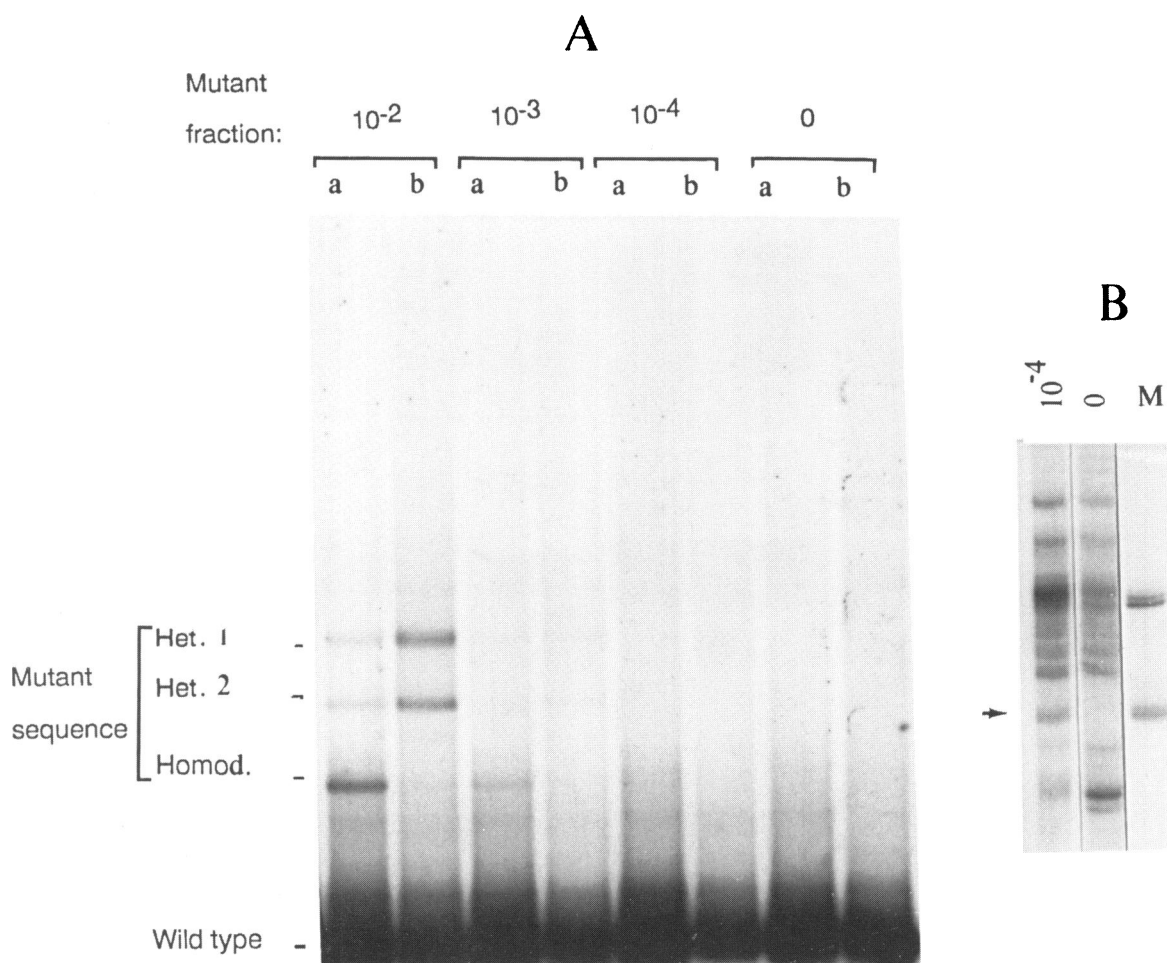


FIGURE 2. Reconstruction experiments to measure the sensitivity of the approach. (A) Samples of  $10^5$  exon 3 wild-type cells (TK6 cell line,  $hprt^+$ ) were mixed with a series of exon 3 mutant cells ( $hprt_{\text{Munich}}$ ) to create mutant fractions of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and 0. The exon 3 sequence was amplified from these cell samples using  $^{32}\text{P}$ -5' end-labeled primers P1 and P2 (Fig. 1) and modified T7 DNA polymerase. The amplified products were analyzed by denaturing gradient gel electrophoresis (DGGE) as both homoduplexes (lanes a, without boiling and reannealing) and heteroduplexes (lanes b, after boiling and reannealing). (B) To detect the lowest limit of sensitivity of the approach, the entire heteroduplex region containing the  $hprt_{\text{Munich}}$  sequences between the wild-type band and the origin of the gel (lane b at  $10^{-4}$ ) was purified from the gel and further amplified with  $^{32}\text{P}$ -5' end-labeled primers. After separation by a second DGGE, one of the  $hprt_{\text{Munich}}$  heteroduplexes present at a mutant frequency of  $10^{-4}$  can be observed (indicated by an arrow), while the other heteroduplex is completely masked by the polymerase noise. The distinctly visible heteroduplex appears with an intensity approximately identical to that of each individual polymerase-induced heteroduplex mutant sequence. M represents the positions of the expected two  $hprt_{\text{Munich}}$  mutant/wild-type heteroduplexes.

lane 7); these were subsequently found to contain mutations, presumably induced by BPDE. The mutant frequency for each mutant band was estimated by calculating the ratio between the radioactivity found in each band and the total radioactivity found in each lane. To determine the kinds and positions of the mutations, each visible band was excised out of the gel, and DNA was isolated from each gel slice, further amplified, and characterized by a second DGGE. In this way, we found that each visible band in Figure 3 could contain either only one or up to two or more mutant/wild-type heteroduplexes, which focused at the same positions in the gel. If a band originally contained only one mutant/wild-type heteroduplex, two major bands were usually observed in the second DGGE: one wild-type and one mutant homoduplex. A band originally containing two or more different mutant/wild-type heteroduplexes would give rise to two or more bands in the second DGGE,

representing different mutant homoduplexes, in addition to the wild-type homoduplex band. The mutant homoduplexes were purified from the gel and sequenced to determine the kinds and positions of the mutations.

### Interpretation of Results

Because sufficiently large numbers of cells were used for the treatment ( $2.4 \times 10^8$  cells per experiment), the descendants of an average of 15,000  $6\text{TG}^R$  initial surviving mutants were selected *en masse* and analyzed per experiment with BPDE. An average of 3000 independent point mutational events were detected in exon 3 of the  $hprt$  gene, representing an average of 15  $6\text{TG}^R$  mutants per base of the total coding region of the  $hprt$  gene and intron sequences flanking each exon in which mutation occurrence may result in the  $6\text{TG}^R$  phenotype. Because a large

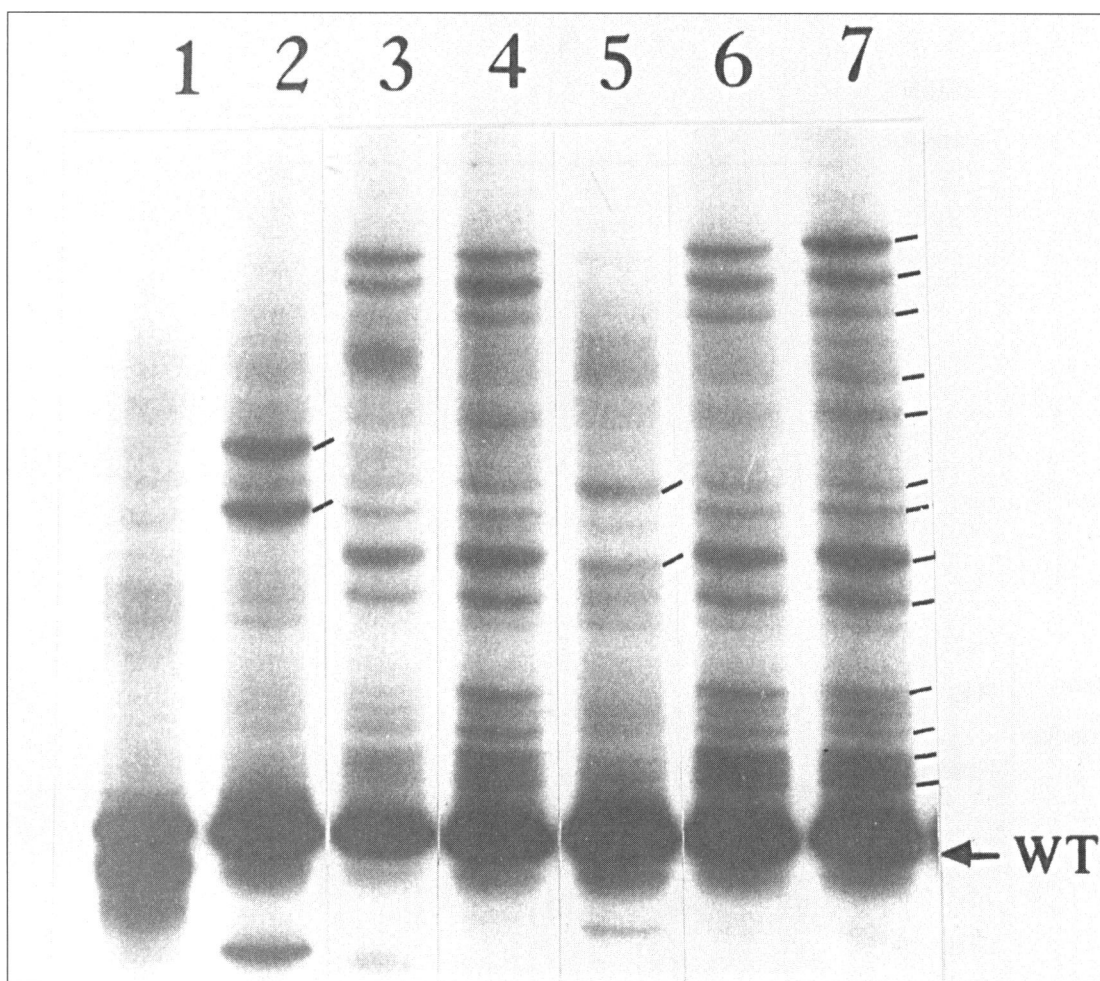


FIGURE 3. Example of denaturing gradient gel (DGG) electrophoresis analysis of mutant sequences induced by benzo[*a*]-pyrene-diol epoxide within the high-temperature melting domain of the *hprt* exon 3 sequence. Exon 3 was amplified from genomic DNA extracted from 6TG<sup>R</sup> cells using first primers P1 and P2. The resultant 224-bp fragment was further amplified using <sup>32</sup>P-5'-end-labeled primers P4 + P2-GC-clamp (Fig. 1). The resulting radioactive 180-bp fragment was gel-purified, boiled, and reannealed to form mutant/wild-type heteroduplexes and analyzed by DGG. The gel was dried and autoradiographed. Lane 1 represents untreated, unselected cells to give the background mutant sequences induced by modified T7 DNA polymerase in this protocol; lanes 2 and 5, untreated but 6TG-selected cells; and lanes 3, 4, 6, and 7, 6-thioguanine-resistant cells after treatment with 40 ng/mL (lanes 3 and 6) and 60 ng/mL (lanes 4 and 7).

number of mutants were analyzed per experiment, the mutational spectra observed in independent experiments are remarkably precise and reproducible (Fig. 3).

The predominant mutations were found to be G to T transversions. This finding is consistent with those of studies previously reported on this chemical, in bacteria (13), rodents (14,15), and, more recently, human cells (16,17) using clone-by-clone analysis. None of these mutations was identical to the modified T7 DNA polymerase-induced mutations within this DNA sequence (8 and unpublished data) or to the background mutations found in stock cultures (this and other studies). Figure 4 compares the mutational spectra induced by BPDE and ultraviolet light (11) in exon 3 of the human *hprt* gene. Ultraviolet light induced five hotspot mutations: two G to A transitions, two A to C transversions, and one A to G transition. Although a run of six Gs (positions 292–297) was found to be the common site for five BPDE-induced hotspots and the two

G to A ultraviolet-induced hotspots, the two carcinogens produced clearly distinct patterns of hotspot mutations with respect to type, position, and frequency within this DNA sequence.

## Conclusion

The combination of high-fidelity PCR and DGGE has permitted us to observe point mutational spectra in cultured human cells with a high degree of precision and reproducibility. Because we used independent and sufficiently large cell cultures for treatment, the variation in individual mutations among independent experiments is consistent with the interpretation that each of them represents a mutational hotspot. This conclusion is consistent with previous observations by Benzer and Freeze (1,2) and Coulondre and Miller (3), who analyzed sufficiently large numbers of mutants in their mutational spectral studies in

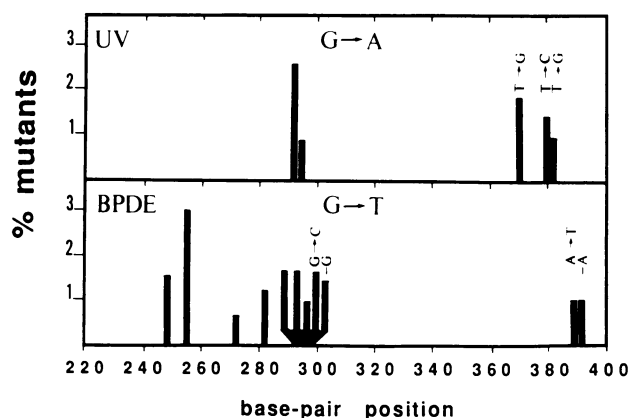


FIGURE 4. Mutational spectra induced in exon 3 of the human *hprt* gene by benzo[a]pyrene-diol epoxide (BPDE) and ultraviolet (UV) light. The patterns of mutations for UV light (11) were determined in a separate study under assay conditions identical to those described here. The horizontal axis represents the exon 3 sequence from positions 220 to 403, which is numbered according to human *hprt* cDNA. For both carcinogens, the frequency of each hotspot mutation relative to 6-thioguanine-resistant mutations is represented by a vertical bar. The predominant hotspots are indicated at the top of each spectrum, and other hotspots are indicated at the tops of each vertical bar.

bacterial systems using clone-by-clone analysis. The novel approach described here may be a useful tool for accurately determining the mutational spectra of other carcinogens in any selectable gene. Our main aim was to devise a tool for observing mutational spectra induced by environmental carcinogens in any DNA sequence independently of phenotypic selection—a condition required for human studies. For this reason, we have been attempting to improve the sensitivity of this new approach. Our current efforts have been focused on *a*) improving the fidelity of PCR DNA amplification and *b*) finding ways first to purify mutant sequences in the original cell samples from the wild type, thus reducing polymerase-induced background mutations. The use of purified mutant sequences as targets for high-fidelity PCR and DGGE analysis can be expected to increase the sensitivity of this new approach.

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